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# Maturational genes upregulation and mitochondrial activity enhancement in mouse in-vitro matured oocytes applying granulosa cells conditioned medium

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## Abstract

The high miscarriage rates that result after transfer of embryos derived from In-Vitro Maturation (IVM) of oocytes necessitates improvements in the processes involved. This study aimed at improving the quality of in-vitro matured oocytes using Granulosa Cells Conditioned Medium (GCCM) as the culture medium. In this work, Germinal Vesicle (GV)-stage oocytes from NMRI mice were collected and cultured using three types of culture media: Base Medium (BM) (control), granulosa cells

conditioned medium 50% (GCCM50) and 100% (GCCM100). After IVM, the mitochondrial activity potential and viability of MII oocytes were evaluated by JC-1 and Trypan blue staining, respectively. Maturational genes expressions of *CyclinB1*, *Cdk1* and *Gdf9* in the control, GCCM50 and GCCM100 samples were analyzed by Real Time PCR. The viability rate of in-vitro matured oocytes was highest in the GCCM50 group. JC-1 staining showed that GCCM50 enhances mitochondrial activity more than the other groups ($P<0.05$). Gene expressions of *Cdk1* and *Gdf9* were higher with GCCM50 treatment, than with control and GCCM100 groups ($P<0.05$), while the expression of *CyclinB1* was not different among the groups. The results indicated that half concentration of GCCM in combination with base medium components, enhances MII and viability rates and mitochondrial activity of mouse immature oocytes.
**Keywords:** In-vitro maturation, granulosa cell, conditioned medium, mitochondrial activity potential, Oocyte.

## Introduction

The in-vitro maturation (IVM) technique is widely used in assisted animal reproduction. It is one of the most efficient methods to produce mature oocytes (Smitz *et al.*, 2011) in procedures such as in-vitro fertilization (IVF), intra cytoplasmic injection (ICSI) and cloning. However, IVM is clinically limited by its poor efficiency in terms of fertilization and viability of the embryos compared to conventional in-vitro fertilization that uses mature oocytes produced in-vivo (Lonergan and Fair, 2016; Gremeau *et al.*, 2012).

It is known that oocytes require maturation of both cytoplasm and nucleus to be able to support successful fertilization and subsequent

embryo development (Kuzmina *et al.*, 2007; Trounson *et al.*, 2001). Nuclear maturation refers to the initiation of meiosis from the germinal vesicle (GV)-stage oocytes and progression to MII. The techniques used to stimulate oocyte development and the culture medium employed, significantly influence oocyte characteristics and growth (Miki *et al.*, 2006; Lenie *et al.*, 2004). There have been, in recent times, considerable research focused on the establishment of optimal conditions for IVM in terms of altering the type of medium used, addition of hormones etc. for better maturation of oocytes (Miki *et al.*, 2006; Nakano and Kubo, 2000; De La Fuente *et al.*, 1999).

In in-vivo follicular development, growth factors and cytokines act as intra-ovarian regulators and gonadotropin modulation is affected by paracrine and autocrine growth factors produced in the ovary (Gilchrist and Thompson, 2007; Sakaguchi *et al.*, 2002). The Granulosa Cells Conditioned Medium (GCCM), obtained from the 3$^{rd}$ passage of surrounding granulosa cells of cultured preantral follicles of mouse, is known to have various cytokines and growth factors like EGF, IGF and TGFβ (Dirnfeld *et al.*, 1997; Malekshah *et al.*, 2006). Given that cytokines and growth factors stimulate resumption of the meiotic process, especially in the activation of the MPF subunits (*CyclinB1* and *CdK1*) that play a dominant role in inducing GVBD stage, it seems logical that conditioned medium derived from cultured granulosa cells could improve maturation outcomes of in-vitro oocyte maturation.

Apart from the factors contained in the granulosa cells, it is known that an increase in the levels of luteinizing hormone (LH) in female leads to resumption of oocyte meiosis. One of leading molecular cascades that

propagate LH induction is a key membrane named epidermal growth factor (EGF) and its subfamily member proteins known as EGF- like peptides (Richani *et al.*, 2014; Park *et al.*, 2004). The increase in in LH levels also results in rapid expression of EGF-like peptides in follicle granulosa cells. These peptides act as transmembrane precursors that diffuse and become attached to the oocyte membrane bound EGF receptors (EGFR). This, in turn, triggers subsequent signaling pathways (Conti *et al.*, 2006). In addition, meiosis resumes following FSH and EGF effects, as too many important inducers, on EGF and FSH receptors.

This research aims to investigate the possibility of improving IVM outcomes through the use of conditioned medium for culture rather than conventional supplementary materials, based on the hypothesis that The GCCM enhances activation and maturation of GV-stage oocytes from mouse germinal vesicles under in-vitro conditions.

## Materials and methods

### Animals and samples preparation

NMRI mice, originally derived from Royan Institute, were housed in a conditioned environment (20-25°C), humidity (40-60%) and 12 h light-dark cycle. The GV-stage oocytes were obtained from 4-6-week-old females ovaries. The procedure was performed on Royan ethical committee guideline.

### Granulosa cells culture and providing the conditioned medium

Preantral follicles (100-120 µm) were mechanically isolated using insulin needles gauge 29, placed into α-MEM supplemented with 10% fetal bovine serum (FBS) and 1% FSH, and incubated at 37 ˚C and 5% $CO_2$. After three days, nonadherent cells were removed after washing twice

with PBS and remained adherent cells were cultured in complete medium until passage 3. While the cultured cells reached to 70% confluency, granulosa cells conditioned medium was collected for using in further IVM.

**In-vitro maturation (IVM) of germinal vesicles**

324 granulosa denuded GV oocytes were obtained from the ovaries of 6-8 weeks old NMRI female mice after mechanical dissection of the antral follicles. The GV-stage oocytes were collected and categorized into three groups based on different culture medium components: 108 GV-stage oocytes in base medium (Control), 108 granulosa cells conditioned medium 50% (GCCM50) and 108 granulosa cells conditioned medium 100 % (GCCM100). α-MEM supplemented by HCG (7.5 IU), FSH (100mIU) and FBS (10%) was used as base medium. For the GCCM50 group, 50% granulosa conditioned medium was added to base medium. In the GCCM100 group, base medium was completely replaced with granulosa conditioned medium (Fig. 1).

The GV-stage oocytes were evaluated by Trypan blue staining for survival assay. Non-Trypan blue positive GV-stage oocytes were randomly divided and incubated in drops of the three media under mineral oil for 24 hours. The in-vitro cultured oocytes were observed by invert microscopy and first polar body extrusion was considered to be the maturation criteria (M II rate).

**Trypan blue staining (oocyte survival assay)**

Trypan Blue (TB) is a diazo dye that is widely used to stain dead tissues or cells. The principle of Trypan Blue Staining (TBS) is that TB is negatively charged and binds only to damaged membranes. Intact cells

allow the passage of very few and select compounds through the membrane, and thus do not absorb TB. Before IVM, GV-stage oocytes treated with TB (0.4%) and found non-positive or viable GV-stage oocytes were selected for in-vitro maturation. Eighteen hours after in-vitro maturation, the oocytes were stained again with TB and non-TB positive M II oocytes were considered viable. By contrast, cells with damaged membranes were stained in a distinctive blue color as readily observed under a microscope.

**Mitochondrial activity potential (JC-1 staining)**

JC-1, a sensitive cationic carbocyanine fluorescence dye that accumulates in the mitochondria, was applied to investigate the mitochondrial potential activity in in-vitro matured oocytes. At low concentrations JC-1 is a monomer and emits green fluorescence (~530 nm), similar to fluorescein, when excited at 490 nm. At higher concentrations, driven by membrane polarization, the dye reversibly forms JC-1 aggregates, which exhibit an emission maximum at ~590 nm (orange color) when the membrane potential is 80-100 mV. JC-1Thus, the color of the dye changes from green to red (Wilding *et al.*, 2001; Smiley *et al.*, 1991). Accordingly, an increase in red/green ratio, indicates an enhancement in mitochondrial potential activity. After 18 h, the oocytes were collected and treated in 0.25 µg/ml JC-1 at 37 for 30 minutes. They were then washed thrice with PBS and finally analyzed using laser-scanning confocal microscope (TCS SP5 II Lecia). The quantification of red/green ratio was analyzed with Image J software.

**Gene expression following in-vitro maturation**

For evaluation of genes involved in resumption of meiosis (*CyclinB1* and *Cdk1*) and oocyte specific gene (*Gdf9*), total RNA was extracted from all samples. Pico Pure Kit (Takara Bio Inc.; Shiga, Japan) was employed for RNA extraction, and then qualified and quantified in each group containing 45 M II oocytes, with a Nano Drop 2000 spectrophotometer (Thermo scientific) 260/280 (1.8-2). Finally, gene expression level was assessed with the qPCR technique following c-DNA synthesis, which was done using the Takamed kit (Takara Bio Inc.; Shiga, Japan) and stored at -20 $^{o}$C until subsequent analysis. Reactions were run with Step One Plus Real Time PCR detection system for qPCR analysis of each 20µl reaction. The primer sequences are provided in table 1. Following this, a melt curve analysis was conducted for each reaction to verify that one product was synthetized with no primer dimer and relative gene expression was calculated using 2-ddct methods. Also, 18S gene considered as “housekeeping gene” and for each reaction a NTC (non-template control) with primer and without cDNA was set for approving the results derived from the other reactions in existence of cDNA.

**Statistical analysis**

Continuous data were initially tested for normal distribution using Kolmogorov-Smirnov test (UNIVARIATE procedure). Data associated with JC-1 and expression of *CdK-1* and *CyclinB-1*, had normal distribution and were analyzed using the GLM procedure; however, the data of *GDF-9* expression did not have normal distribution and were analyzed using Kruskal Wallis test. Binary data including the proportion of GV and MII were analyzed using GENMOD procedure including function link logit in the model. The LSMEANS statement was used for implementation of multiple comparisons. All analyses were conducted in

SAS (User's guide version 9.4: statistics. Cary, NC: SAS Institute; 2013 SAS). Data are presented as proportions or mean ± SEM. Differences with $P < 0.05$ were considered significant.

## Results

### Mouse GVs in-vitro maturation

Fig. 2 shows the proportion of oocytes that have released their polar bodies and reached the MII stage after 18 hours culture. In this case, the maturation rates of both GCCM100 (77.78%; odds ratio [OR] = 2.06, 95% confidence interval [CI] = 1.13-3.75; P = 0.02) and 50 75.93%; OR = 1.86, 95% CI = 1.03-3.34; P = 0.04) groups were significantly higher than control (62.96%) ($P < 0.05$). In this regard, the difference of MII rate between GCCM50 and GCCM100 groups ($P > 0.05$) was insignificant. At various intervals from the onset of incubation, oocytes were observed by invert microscopy and evaluated for morphological changes in nucleus and polar body as criteria for nuclear maturation of GV-stage oocytes.

### Trypan Blue Staining (oocyte viability)

TBS was applied to assess the viability of GV-stage oocytes and then of in-vitro matured oocytes. Following the first staining, the viable germinal vesicles were selected for further in-vitro maturation. The next staining by TB was carried out to evaluate oocytes matured in-vitro (Fig. 3).

The TBS technique showed that the highest viability rate of in-vitro matured oocytes occurred in the samples that used 50% granulosa conditioned media (GCCM50) and this was comparable to the control

BM group (OR = 2.67, 95% CI = 1.09-6.52; P=0.03) and non-comparable with GCCM100 group.

**Mitochondrial potential activity (JC-1 Staining)**

The mitochondrial potential activity (MPA) was detected by confocal microscopy (Olympus Invert microscope) measured by Image J software (Image J 1.46r, Java 1.6.0_20) in in-vitro matured M II oocytes. MPA was analytically higher in GCCM50 than both control and GCCM100 groups (P <0.05), but total concentration of granulosa conditioned media (GCCM100) did not have comparable effects on IVM rate against BM group (P<0.05) (fig.4).

**Expression level of genes following in-vitro maturation**

The relative mRNA expression of maturation promoting factor (MPF) complex showed that the level of *Cdk1* expression in GCCM50 was significantly higher than control (BM) group (P<0.05), but there was no significant difference between GCCM100 with GCCM50 and control groups (P>0.05) (Fig. 5). However the expression of CyclinB1 gene was not different among all groups (P>0.05). *Gdf9* gene expression increased for both 50 and 100% of granulosa conditioned media; that in the GCCM50 group was higher than those of both control and GCCM100 groups (P<0.05). In addition, *Gdf9* expression was higher in GCCM100 than control group (P<0.05) (Fig. 5).

## Discussion

The present study is the first report on the ability of granulosa cells conditioned medium (GCCM) to support in-vitro maturation (IVM) of GV-stage oocytes to MII stage. The results showed that GV- stage oocytes

isolated from 6-8 weeks old NMRI female mice, 18h after culture in half concentration of GCCM (GCCM50 group), showed better maturation rates than both BM (without GCCM) and GCCM100 (full concentration of GCCM) groups. The benefits of granulosa cells on oocyte maturation and subsequent embryo development have been demonstrated earlier (Malekshah *et al.*, 2006; Kobayashi *et al.*, 1992). Malekshah and his co-workers investigated in-vitro culture of mouse embryos in medium conditioned with human granulosa cells and showed a small improvement in outcome, albeit not comparable to co-culture. The effectiveness of granulosa cells to support embryo development until the blastocyst stage was reported by Madea et al (Myers *et al.*, 1994). Among the growth factors that have been used to induce better maturation and fertilization outcomes in oocytes, the Epidermal Growth Factor (EGF) has been known to induce meiotic resumption in various mammalian species like rat (Lanuza *et al.*, 1998), mouse (Das *et al.*, 1992), Cattle (Nandi *et al.*, 2002), Pig (Sirotkin *et al.*, 2000), dog (Bolamba *et al.*, 2006) and buffalo (Singhal *et al.*, 2009).

This study aimed to investigate the effects of granulosa conditioned medium on upregulation of maturation genes and enhancement of mitochondrial activity in mouse oocytes matured in-vitro. Here, we showed that GCCM induces meiosis resumption and improves the GVBD rate. Interestingly, using of base medium combined with granulosa cells conditioned medium, significantly improved in-vitro maturation rates of mouse germinal vesicles.

To gain better understanding of the GCCM improvement mechanism at the nuclear level, the expression levels of both subunits (*CdK1*, *CyclinB1*) of maturation promoting factor (MPF) were assessed. *CdK-1* mRNA was upregulated in GCCM50 group more than the control (Fig.

5). But the level of *CyclinB1* gene expression was not different among all groups.

The results of the present study showed that IVM rate increased in the presence of granulosa cells conditioned medium as compared to the control group that did not contain granulosa cells. It seems that the presence of growth factors like EGF and IGF1, is responsible for better in-vitro oocyte maturation. The synergistic effects of FSH along with granulosa cells secretions in GCCM50, lead to increased effect of the medium on meiosis resumption. Although there are a few reports mentioned about the inhibitory effect of IGF1 on oocyte maturation (Guler *et al.*, 2000), some studies have proposed positive effects of IGF on oocyte stimulation. Liang et al., showed that IGF activates the MAPK followed by PI3K/Akt induction after attaching to its receptors on the surface of the oocyte. These events activate the MPF after removing the stopping forces from *Cdc2* tracked by P90rsk activation and Myt1 inhibition. To the role of IGF1 role on in-vitro maturation of oocyte in Xenopus has also been mentioned in literature (Schmitt and Nebreda, 2002).

The results of the present study agrees with earlier reports that granulosa cells conditioned medium have factors like IGF1, which could induce and sustain oocyte meiosis. The other effective factor is EGF which provides a large amount of granulosa cells conditioned medium. EGF induces another ERK 1/2 and then steroidogenesis and MOS signaling pathways (Shimada *et al.*, 2006). The last factors activate MPF and meiosis resumption through contact with oolema. Richani et al., firstly reported mitochondrial activity improvement using of EGF-like peptide. Our study showed similar results in the upgradation of mitochondrial membrane potential activity by the granulosa cells

secretions. It seems that this effects may enhance adjacent to base medium composed of FSH in GCCM50 group. We also analyzed the gene expression of oocyte specific marker, *Gdf9*, in all groups. *Gdf9* was found to be upregulated in GCCM50 medium more than in control and GCCM100 group. GDF9 as a growth factor has a robust impact on proliferation and viability of granulosa cells and follicle. *Gdf9* upregulation mentions to higher responsivity of oocyte to granulosa cells conditioned medium components. It seems that raising the oocyte activity leads to raise the quality and viability of oocyte. This statement approved by progressive oocyte viability in both GCCM50 and GCCM100 groups compared to BM group. This mentions to an influential helpful role of granulosa cells conditioned medium on oocytes during IVM.

Since mitochondria is the major source of ATP, the energy molecule, and plays an important role in cellular metabolism, it's membrane potential activity is closely related to both in-vivo and in-vitro developmental potential of oocyte and embryo (May-Panloup *et al.*, 2007). The quantity, localization and activity of mitochondria in oocyte have been shown (Wilding *et al.*, 2001). In the present study, we used JC-1 staining methods to show a marked difference in mitochondrial membrane potential activity between the BM and GCCM50 groups.

To conclude, this work showed that maturation and viability rates and mitochondrial membrane potential activity of in-vitro cultured oocytes are significantly enhanced on using a combination of granulosa cells conditioned medium as seen in the GCCM50 group. It was also shown that the presence of FSH in the base medium in the GCCM50 sample shows synergetic effects on signaling pathways and oocyte maturation.

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## Conflict of Interest:

None.

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**Table 1.** Sequences of primers for Real Time PCR test.

| Gene | Primer | Primer Length (bp) |
|---|---|---|
| *Gdf9* | F: TGAACAACTCTGCCTCTTCC | 22 |
| | R: ATGCTAAACACTCCGTCCTC | 22 |
| *CdK-1* | F: GACAAAGGAACAATCAAACTGG | 22 |
| | R: GCAAATATGGTCCCTATACTCC | 22 |
| *CyB1* | F: AGGGTCACTAGGAACACGAAA | 21 |
| | R: TATTACCAATGTCTCCAAGAGCAG | 24 |
| *18S* | F: TTGACGGAAGGGCACCACC | 19 |
| | R: GCACCACCACCCACGGAAT | 19 |

**Fig. 1:** Designing of the groups based on different concentration of granulosa cells conditioned medium (GCCM) in IVM media.

**Fig. 2:** Percentage of polar body released oocytes (M II rate) in all control (BM) and Experimental groups (GCCM50 and 100). *significant with other groups: P<0.05.

**Fig. 3:** Trypan blue staining to select the viable germinal vesicles for IVM and viable in-vitro matured oocytes (M II oocytes). Viable GV (A) and dead GV (B) before IVM, C) viable MII oocyte after maturation in base medium (control group), viable MII oocytes after maturation in GCCM100 (D) and GCCM50 (E). Graph indicates different viability rate after IVM; A with a: P = 0.012.

**Fig. 4:** JC-1 staining for mitochondrial membrane potential activity; red to green: increasing of membrane potential activity (MPA). Green: JC-1 monomeric form (low intensity), Red: JC-1-aggregated form (high intensity). Graph; *significant with other groups: P < 0.05.

**Fig. 5:** Gene expression level of *CdK1*, *CycB1* and *Gdf9* in in-vitro matured oocytes (M II) of all control (BM) and experimental (GCCM50 and 100) groups (*significant with BM) (**significant with BM and GCCM50)(P<0.05).

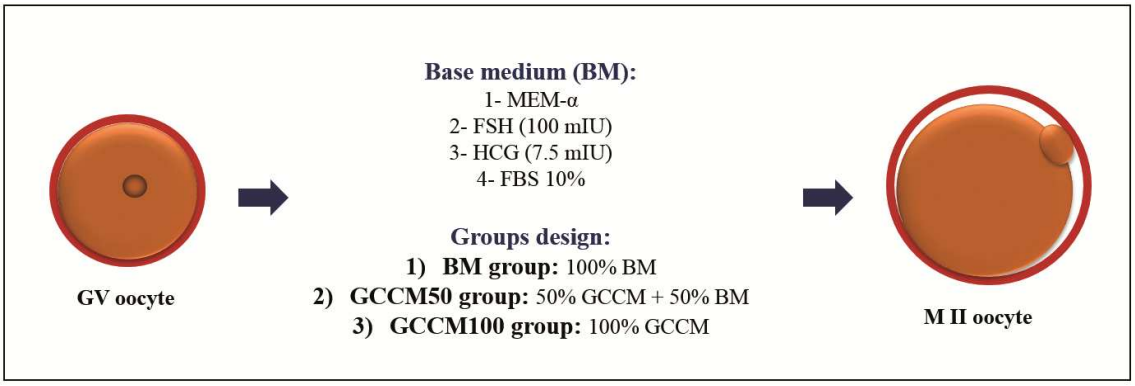


Figure 1

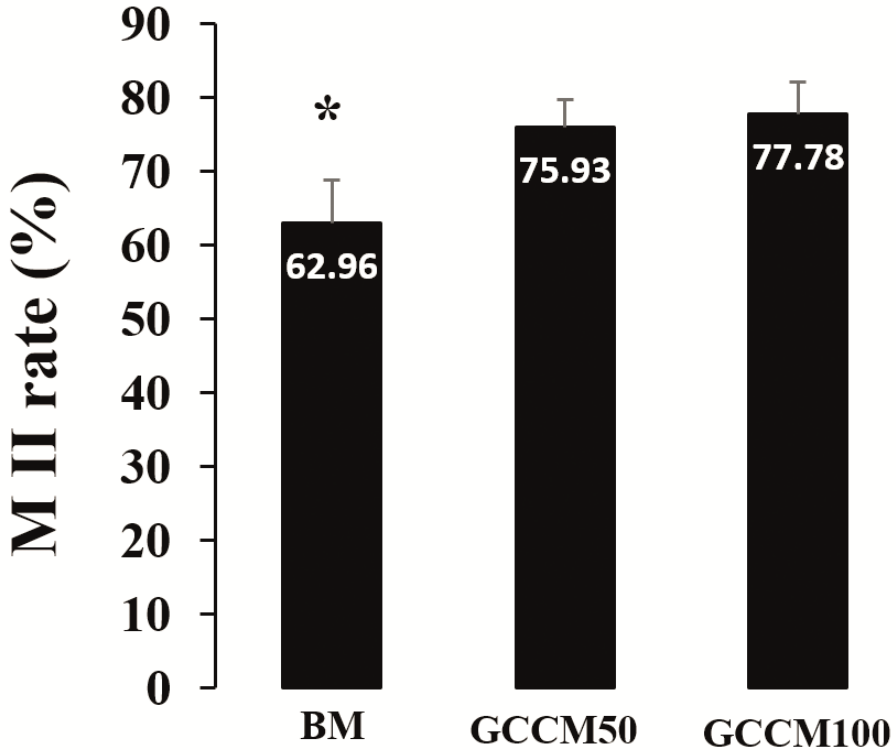


Figure 2

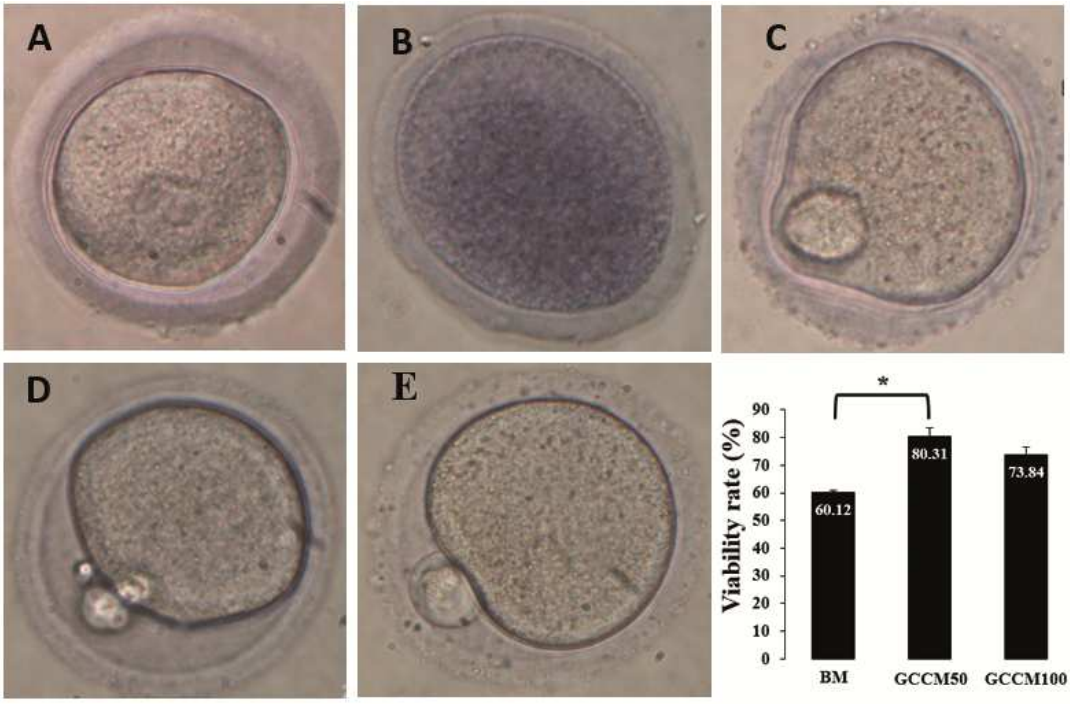
A
B
C
D
E
*
Viability rate (%)
90
80
70
60
50
40
30
20
10
0
60.12
80.31
73.84
BM
GCCM50
GCCM100

Figure 3.

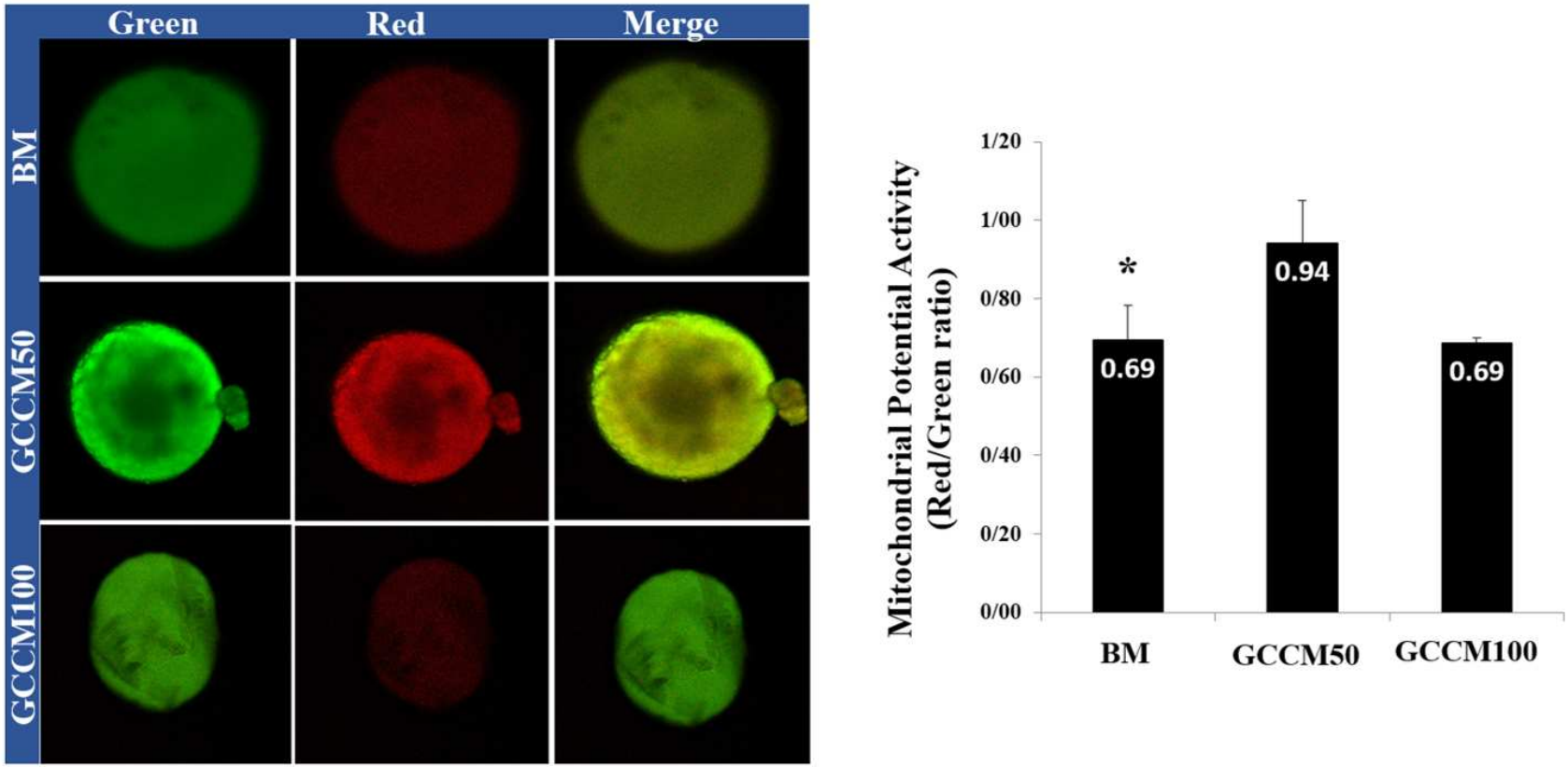
Green
Red
Merge
BM
GCCM50
GCCM100
Mitochondrial Potential Activity
(Red/Green ratio)
1/20
1/00
0/80
0/60
0/40
0/20
0/00
*
0.69
0.94
0.69
BM
GCCM50
GCCM100

Figure 4.

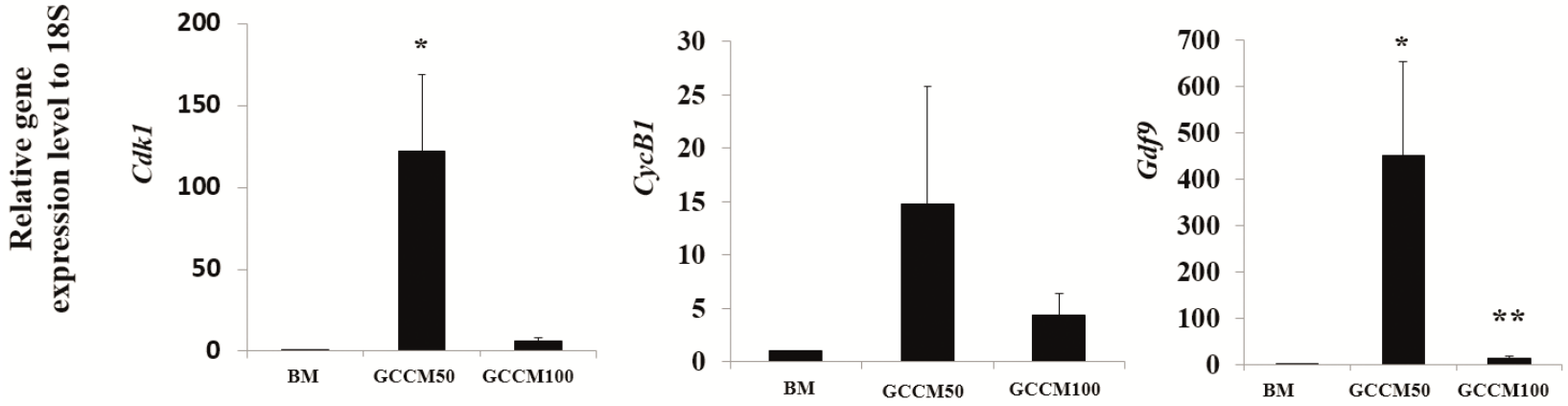
Relative gene expression level to 18S
Cdk1
200
150
100
50
0
*
BM
GCCM50
GCCM100
CycB1
30
25
20
15
10
5
0
BM
GCCM50
GCCM100
Gdf9
700
600
500
400
300
200
100
0
*
**
BM
GCCM50
GCCM100

Figure 5.